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Enhanced Production of Recombinant Extractable Antigen (EA1), an Extracellular Protein and its use in Detection of Spores of *Bacillus anthracis*, the Causative Agent of Anthrax

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ABSTRACT

Detection of spores of *Bacillus anthracis*, the causative agent of anthrax in human and animals in environment is cumbersome due to the presence of spores of other closely related *Bacillus* species. The Extractable Antigen 1 (EA1), an extracellular protein is considered as a biomarker for detection of *B. anthracis* spores. In the present work, we have cloned and expressed the recombinant EA1 protein in soluble form in *Escherichia coli*. Optimisation of culture conditions and cultivation media was carried out to achieve enhanced soluble expression of recombinant EA1 protein. Further, the batch fermentation process was also developed using optimised conditions for scale up production of recombinant EA1 protein. The final yield of protein purified employing affinity chromatography was 42.64 mg/l of culture during batch fermentation process. The polyclonal antibodies were raised against recombinant EA in rabbit and mice and used to develop an ELISA for detection of *B. anthracis* spores. The specificity of the developed assay was ascertained with spores of other *Bacillus* species. The results corroborated that the EA1 could be a suitable biomarker for detection of *B. anthracis* spores.

Keywords: *Bacillus anthracis* spores; *Escherichia coli*; Extractable antigen; Bioreactor; Purification; ELISA

1. INTRODUCTION

Anthrax is a potentially fatal disease of animals and human caused by a Gram positive and spore forming bacterium, *Bacilllus anthracis*. Anthrax is considered as a potential disease of biological warfare[1]. Besides, several cases of human and animal anthrax are reported from, any agrarian countries. Hence, it is imperative to develop the simple methods for detection of *B. anthracis*.

B. anthracis harbours two mega plasmids, pXO1 (182 kb) and pXO2 (95 kb), which are essential for its virulence[2]. *B. anthracis* produces a capsule during its growth in animal or human host which evades the immune system[3]. However, in culture broth or in absence of pXO2 plasmid, capsule is not formed and a cell wall with highly patterned ultra structure layers is formed[4]. These layers that cover the entire bacterial cell surface are referred to as the surface layers or S-layers[5,6]. These S-layers are produced by non-covalent, entropy-driven self assembly of identical protein or glycoprotein subunits and have strong interaction with the cell wall[7,8]. Among the S-layer proteins, extractable antigen (EA1) is found abundantly on the surface layer of vegetative form as well as on the surface of *B. anthracis* spores[9]. It has also been established that EA1 is not the integral component of *B. anthracis* spores but is present as a contaminant during preparation of spores[9]. However, EA1 is highly spore associated protein and remains bound on the spore surface even after rigorous washings[10]. Hence, it can be a suitable biomarker for detection of *B. anthracis* spores. Earlier studies also established the use of EA1 in the *B. anthracis* detection[10-12].

Detection of *B. anthracis* spores is established by employing various methods including culturing followed by biochemical characterisation, immunofluorescence[13], ELISA and other immunological systems[14-17], PCR[18], and surface plasmon resonance[19]. However, these methods are time consuming, laborious and require sophisticated laboratory and infrastructure. Hence, a simple, sensitive and affordable detection system is required for *B. anthracis* spores.

The recombinant proteins for use in detection and diagnosis are generally produced using either prokaryotic or eukaryotic expression systems. *E. coli* is the most commonly used host for recombinant protein production due to its fast growth rate and well-characterised genetics[20,21]. Hence, the objective of the present study was to produce recombinant EA1 protein with high yield and develop an effective diagnostic test for detection of *B. anthracis* spores. The recombinant protein expression level in *E. coli* are affected by different parameters such as cultivation media, temperature, duration of induction, concentration of inducer and mode of cultivation. Hence, these parameters need to be optimised to enhance the yield of recombinant proteins. Recombinant protein production in *E. coli* has been extensively studied by batch fermentations using complex medium. Affinity chromatography is generally

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employed for purification of the histidine tagged recombinant proteins[20,22].

In this study, the rEA1 was cloned, expressed in *E. coli* and scaled up using bioreactor. The affinity purified protein was used to produce antibodies in rabbit and mice. An antigen capture ELISA was developed for detection of *B. anthracis* spores using the purified antibodies.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Bacillus anthracis Sterne, a non-pathogenic but toxigenic strain and other *Bacillus* strains viz. *B. cereus* (ATCC 11778), *B. subtilis* (ATCC 6633), *B. globigii* (ATCC 49760), *B. thuringiensis* (MTCC 4714), *B. mycoides* (MTCC 645) and *B. pumilus* (MTCC 1640) were available in our laboratory.

2.2 Cloning of EA1 Gene

Genomic DNA was extracted from the overnight grown culture of *B. anthracis* Sterne in LB broth (Jackson et al. 1997). The primers; EA1-F (5′-C A T G G A T C C G C A G G T A A A T C A T T C C C A G -3′, the *BamH1* site underlined) and EA1-R (5′-ATACTCGAGTAGATTTGGGTTATTAAGAAGG -3′, the *XhoI* site underlined) were used to amplify the *eag* gene (GenBank accession no. X99724.1). The primers were custom made from Eurofins, India. High fidelity DNA polymerase (NEB, USA) was used for PCR amplification of the target sequence of 2499 bp. The PCR amplification conditions were: initial denaturation for 2 min at 94 °C followed by 30 cycles of 1 min at 94 °C (denaturation), 1 min at 55 °C (annealing) and 2 min at 72 °C (extension) with a final extension of 10 min at 72 °C. The PCR product was purified. The pET32a+ expression vector (Novagen, USA) containing histidine tag was used in cloning. The restriction enzymes, *BamH1* and *Xho1* were used to double digest the PCR product and the vector. The digested products were electrophoresed (1% agarose), purified by gel extraction and quantified by Nanodrop Spectrophotometer (Corbett, Australia). For ligation, a 4:1 molar ratio of insert to vector was used along with T4 DNA ligase (NEB, USA) at 16 °C for 12 h. The ligated plasmid was transformed into chemically competent *E. coli* DH5α and subsequently to *E. coli* BL21 (DE3), the expression host.

2.3 Expression of rEA1 Protein

Expression of rEA1 was initially carried out at shake flask culture by inoculating 2% (v/v) of overnight (16 h) grown culture. The cultures grown in LB broth (10 ml test tube) at 37 °C were induced with 1.0 mM IPTG at an OD_{600} of 0.6. After 4 h of incubation at 180 rpm in an incubator shaker (Kuhner AG, Switzerland) and 37 °C, the 1 ml cultures were centrifuged at 8000 rpm for 10 min at 4 °C. To the washed pellet, 100 µl cell lysis buffer was added and centrifuged at 8000 rpm for 10 min at 4°C. About 10 µl of supernatant sample was analysed by SDS-PAGE, (12% gel) in duplicate. One gel was stained with Coomassie brilliant blue and the other was electroblotted on to polyvinylidene fluoride (PVDF) membrane (Millipore, USA). Histidine tag at N-terminal in the rEA1 protein was confirmed by Western blotting with anti-His antibody (Sigma, USA).

2.4 Effects of Different Parameters on rEA1 Protein Expression

Protein expression level depends on various factors including IPTG concentration, post induction time, post induction temperature and culture media. For optimisation of inducer concentration, the cells grown in LB medium were induced with different IPTG concentrations (0.1, 0.25, 0.5 and 1.0 mM) for 4 h at 37 °C. Induction duration was optimised by inducing the cultures with 1 mM IPTG for 4 and 16 h. The post induction temperature of culture was optimised at 16 °C, 25 °C and 37 °C. After centrifugation, all the cultures were analysed by SDS-PAGE for protein expression. To evaluate the effect of culture media, five types of media viz. Luria Bertani (LB) Broth, Super Broth (SB), SOB, SOC and Terrific Broth were used for rEA1 expression. All five types of media (50 ml each) in duplicate containing 100 µg/ml ampicillin were inoculated with 1 ml of 16 h grown culture and incubated at 37 °C, 180 rpm. When culture OD_{600} reached to 0.6-0.8, 1.0 mM IPTG was added for induction at 37 °C for 4 h. After centrifugation at 8000 rpm for 20 min at 4 °C, the pellets were collected and frozen at -20 °C.

2.5 Bioreactor Studies

The rEA1 was scaled up using 5 litre bioreactor (Bioflo 3000: New Brunswick, USA) under conditions described earlier [17, 23]. The culture (5 % v/v) was inoculated in bioreactor vessel containing 4.5 l of TB medium with antibiotic and run with initial condition of temperature 37 °C, pH 7.0, agitation 100 rpm and air 2.5 LPM. The dissolved oxygen (DO) concentration was kept at 20–30% of air saturation by varying agitation rate between 100 and 400 rpm. The cultivation pH was controlled between 6.8 and 7.0 using 25 % ammonia solution or H_3PO_4, whenever required. Antifoam was added to prevent foaming during cultivation. The cultures were grown till mid log phase (4 h post inoculation) and inducer (0.1 mM IPTG) was added for protein expression. Cells were recovered 16 h post induction using centrifugation at 8000 rpm for 30 min at 4 °C and stored at -20 °C.

2.6 Purification of rEA1 Protein

For purification of rEA1, cell pellet was washed twice with distilled water and washed pellet was further dissolved (1: 10 w/v) in cell lysis buffer. The cells were sonicated using sonicator (Biologics, USA) for a total of 10 min (9 s on and 9 s off). After sonication, homogenate was clarified by centrifugation at 8000 rpm for 45 min and the supernatant was collected for further purification. Immobilised metal affinity chromatography was used for purification of rEA1. After filtration through 0.45 µm membrane filter, the protein was purified using a 5 ml pre-packed Ni-NTA affinity chromatography column (Qiagen, Germany) on AKTA Explorer chromatography system. The column was pre equilibrated using lysis buffer (10 CV) at a flow rate of 1 ml/min before sample loading. After loading the sample, column was washed with wash buffer containing 50 mM imidazole and the protein was eluted in elution buffer containing 250 mM imidazole. All the eluted fractions were analysed by SDS-PAGE. Affinity chromatography purified protein was diafiltered in PBS containing 10% glycerol.

The protein concentration was assessed using BCA reagent (Thermo, USA).

2.7 Analytical Methods

The culture growth was monitored by the optical density (OD_{600}) measurement using spectrophotometer (Thermo, USA), and wet cell weight. For wet cell weight measurement, 10 ml of culture was centrifuged in pre-weighed 15 ml tubes at 8000 rpm for 20 min, and weight of pellet was taken after decanting the supernatant.

SDS-PAGE was used to check expressions and purity of EA1 after staining with Coomassie Brilliant Blue. For Western blot analysis, the lysate containing EA1 as well as affinity chromatography purified EA1 was separated on 12 % SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in PBS + 5 % skimmed milk protein (SMP) at 4 °C for 16 h. Blocked membranes were washed thrice for 5 min with PBS-T [PBS + 0.05 % Tween-20 (v/v)] and incubated at 37 °C for 1 h with anti-His antibodies (1:5000 dilution in PBS+1 % SMP). After washing, the membranes were incubated with rabbit anti-mouse IgG HRP conjugate (1:10000) for 1 h at 37 °C. The membrane was developed in diaminobenzidine (DAB)/ H_2O_2 substrate solution for 15 min at 37 °C.

2.8 Production and Characterisation of Anti EA1 Rabbit and Mice Polyclonal Antibody (pAb)

For production of polyclonal antibodies, two male New Zealand white rabbits and six female BALB/c mice (8 week old) were used according to the Institutional Animal Ethics Committee (IAEC). The animals were immunised with purified rEA1 (200 µg/ rabbit/dose and 20-25 µg/mouse/dose) with Freund complete adjuvant for priming followed by two booster doses with incomplete adjuvant subcutaneously on days 21 and 42. Rabbit and mice were bled before immunisation to collect serum. Final bleeding was carried out on day 60 and the sera were stored at -20 °C for further use.

To evaluate the reactivity of rabbit and mice sera, Western blot analysis was carried out. Purified rEA1 was run on SDS-PAGE and transferred on PVDF membrane and further steps were carried out as mentioned above with incubation in anti-EA1 mice antiserum 1:2000 dilution and anti-EA1 rabbit antiserum 1:2000 dilution, respectively. The rabbit-anti-mouse IgG and goat- anti-rabbit IgG HRP conjugated secondary antibody was used for rabbit and mice sera, respectively. The protein bands were visualised by incubating in substrate DAB/ H_2O_2 for 2 min at room temperature.

To determine the antibody titre of rabbit and mice sera in indirect ELISA, rEA1 (200 ng/well) in coating buffer was added to each well of Maxisorp ELISA plates and incubated for 16 h at 4 °C. After washing the plates with wash buffer (PBS with 0.1% Tween - 20), 300 µL of blocking buffer (5% SMP in PBS, pH 7.2) was added to each well, and incubated for 1 h at 37 °C. The plates were again washed and 100 µl of two-fold serially diluted mice or rabbit antiserum ranging from 1:1000 to 1: 1024000 in 1% SMP in PBS was added to each well and kept for 1 h at 37 °C. The plates were washed again and the specifically bound antibodies were detected by adding 100 µl/well of HRP-conjugated anti-mice IgG (1:4000) or HRP- conjugate anti-rabbit IgG (1:10,000) in respective wells for 1 h at 37 °C. To each well, 100 µl of 3,3',5,5'-tetramethylbenzidine (Sigma, USA) was added after three washings of plates. After 10 min incubation at 37 °C, the reaction was stopped with 50 µl of 2.5 N H_2SO_4 and plate was read at 450 nm with ELISA plate reader (BioTek Instruments Inc, USA). Serum of rabbits collected before immunisation was used as control. Each serum sample was tested in triplicate. Antibody titre was expressed as reciprocal of the end point dilution.

2.9 Spore Production and Characterisation

For spore production, single colony of various *Bacillus* species were inoculated in BHI medium and incubated for 16 h at 37 °C on a shaker at 170 rpm for the preparation of inoculum. This inoculum (10 % v/v) was used to inoculate in shake flask with 500 ml Difco Sporulation Medium. About 1 ml of Ca $(NO_3)_2$ 1 M, $MnCl_2$ 10 mM and $FeSO_4$ 1 mM was added during inoculation. The culture was incubated at 37 °C on an incubator shaker at 170 rpm for 72 h and analysed under microscope for spore formation.

To further confirm the presence of *B. anthracis* spores, 10 µl of heat lysate of 10^7 spores/ ml of Sterne was run on 12 % denaturing gel and transferred to PVDF membrane. After blocking with 5% SMP as described above, the membranes were incubated with anti-EA1 rabbit or mice antibodies. After treating with goat anti-rabbit or rabbit anti- mice IgG HRP conjugate for 1 h at 37 °C, the membranes were developed with DAB/H_2O_2 substrate solution for 15 min at 37 °C.

2.10 Development and Evaluation of Antigen Capture ELISA for Detection of *B. anthracis* EA1

Antigen capture ELISA was developed to detect EA1 for *B. anthracis* spores. Ninety-six-well ELISA plates were coated with purified rabbit anti-EA1 antibodies (0.2 µg/100 µl/ well) in coating buffer at 4 °C for overnight. Plates were then washed three times with PBS-T followed by incubation at 37°C in blocking buffer for 1 h. The plates were then washed with PBS-T (0.1 % Tween-20 in PBS, pH 7.4) and incubated with different concentration of purified EA1 starting from 200 ng to 0.39 ng / well in PBS containing 1% SMP. The plates were washed again after 1 h incubation at 37 °C and added 0.2 µg/100 µl/ well anti EA1 mouse polyclonal antibody in 1% SMP (in PBS) for 1 h at 37 °C. Rabbit anti-mouse HRP-conjugate (1:4000) was added to the plate (100 µl/well). Plate was incubated at 37 °C for 1 h followed by washing with PBS-T. About 100 µl TMB substrate solution was then added per well and kept at 37 °C for 10 min. The plates were read at 450 nm after adding the stop solution. Each concentration of antigen was tested in triplicate and the mean OD for each sample was determined.

The ELISA was evaluated for detection of spore of *B. anthracis* (Sterne strain). For this purpose, instead of EA1 antigen, different numbers of spores were tested for presence of EA 1 on *B. anthracis* spores and the rEA1 antigen was used as a positive control. Spores were diluted in 1 % SMP in PBS to prepare the sample. The specificity of the ELISA was

evaluated with spores of other *Bacillus* species including *B. cereus*, *B. subtilis*, *B. globigii*, *B. thuringiensis*, *B. mycoides* and *B. pumilus*.

3. RESULTS

3.1 Preparation of Recombinant Host and Expression of EA1

The DNA of *B. anthracis* Sterne was extracted and used for PCR amplification of *eag* gene (2499 bp). The PCR product was purified and cloned into pET32a+ vector using *BamHI* and *XhoI* restriction enzymes and transformed in *E. coli* DH5α. The cloning of PCR product into *E. coli* DH5α was confirmed by reverse restriction digestion. The plasmid from DH5α was purified and transformed into expression host *E. coli* BL21 (DE3). The expression of rEA1 was confirmed by SDS PAGE (Fig. 1(a)) and Western blotting (Fig. 1(b)).

3.2 Optimisation of Culture Conditions for Expression of rEA1

Varying IPTG concentrations starting from 0.1 mM to 1.0 mM did not show much effect on protein expression. Hence, 0.1 mM concentration of IPTG was used in further experiments. Induction time of 16 h at 16 °C was found optimum for protein expression. Expression of protein at 16 °C was higher than at 25 °C and 37 °C and majority of the protein was in soluble form.

For optimum expression of rEA1, five different media were tested at shake flask culture. TB media yielded the highest OD of 2.36 followed by the OD values of 1.28, 2.2, 2.0 and 2.04 in LB, SB, SOB and SOC media, respectively. TB medium was found better in terms of protein expression in comparison to other media (Fig. 2). It was also observed that induction at 1.0 OD_{600} resulted in higher level of protein expression as compared to induction at 0.6 OD_{600} in TB medium.

The concentration of EA1 present in soluble and insoluble fractions in TB medium was analysed by BCA method. The soluble protein fraction was 70 %, whereas insoluble fraction was 30 % at 16 °C for 16 h induction.

3.3 Scale up Production of rEA1 using Bioreactor

The rEA1 was scaled up by batch fermentation in 5 l bioreactor. Various parameters optimised at small scale were employed for this purpose. For bioreactor, cultures dissolved oxygen (DO) level above 20% was maintained. In batch fermentation, a wet cell weight of 10.5 g/l was obtained.

3.4 Purification and Characterisation of rEA1

For purification of the protein, the pellet was lysed and processed further for laboratory scale purification using immobilised metal affinity chromatography. The EA1 could bind onto the resin and the impurity in protein was washed using wash buffer. The protein was eluted with elution buffer containing 250 mM imidazole, pH 8.0. The protein yield of shake flask and batch fermentation process was 32.64 mg/l and 42.64 mg/l of culture, respectively. The real time profile of purification has been shown in Fig. 3. The SDS–PAGE analyses confirmed the recovery of protein by affinity purification (Fig. 4(a)). The purified rEA1 was reactive in Western blot using anti-His antibody (Fig. 4(b)).

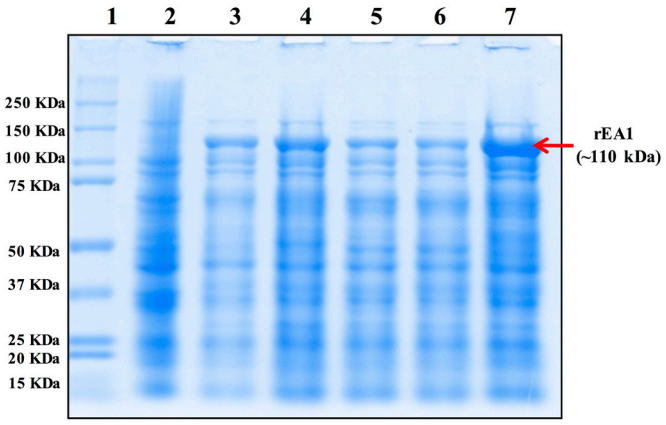

Figure 2. SDS-PAGE analysis showing effect of different media on expression of rEA1. Lane 1: Protein markers; lane 2: Un-induced culture; lane 3: LB medium; lane 4: SB medium; lane 5: SOB medium; lane 6: SOC medium; lane 7: TB medium.

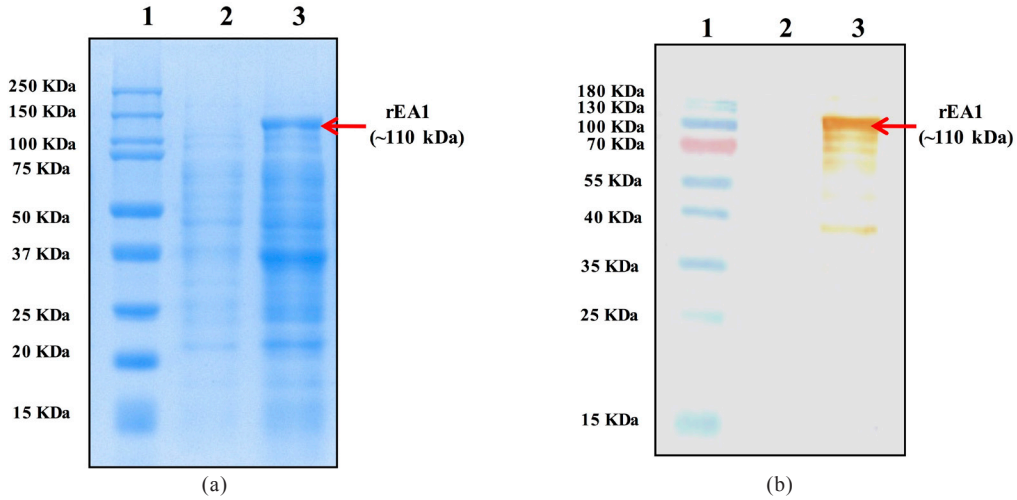

Figure 1. Expression profile of rEA1. (a) SDS-PAGE profile of expressed protein. Lane 1: Protein markers; lane 2: Un-induced culture; lane 3: induced culture containing rEA1. (b) Western blot analysis to confirm expression of rEA1. Lane 1: Protein markers; lane 2: Un-induced culture; lane 3: Expressed recombinant protein.

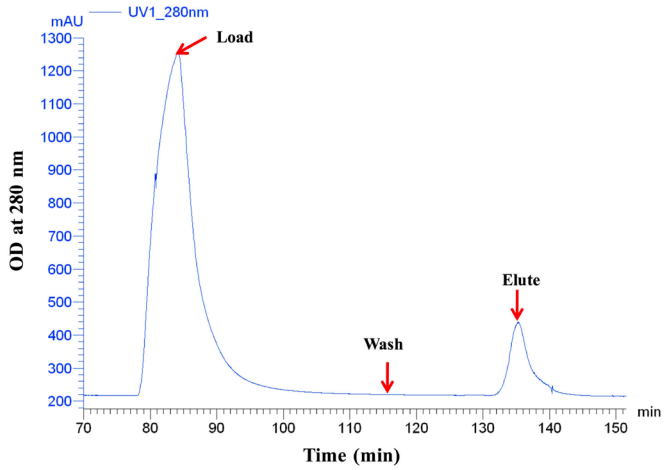

Figure 3. Chromatogram depicting purification of rEA1 using a 5-ml pre-packed Ni-NTA column on AKTA explorer chromatography system.

3.5 Determination of Anti-EA1 Rabbit and Mouse Polyclonal Antibody Titre

The rEA1 with Freund's adjuvant developed higher levels of EA1 specific antibodies titre. The mean of the pre-immune sera (control sera) sample, plus three standard deviations (SD) was taken as the cut-off value. The antibody raised in rabbit and mouse exhibited a titre of 1:1024000 and 1:512000, respectively with rEA1 in indirect ELISA.

3.6 Spore Production and its Characterisation

In DSM medium, >95% spores were produced with spore count of 10^8 spores/ ml. Spores were characterised by microscopic observations. Anti-EA1 polyclonal antibodies were evaluated in Western blot for their potential in detection of spores of *B. anthracis*. In Western blotting, spores of *B. anthracis* Sterne were found reactive with anti-EA1 rabbit antibody (data not shown). Hence, EA1 can be used as a target for detection of *B. anthracis* spores.

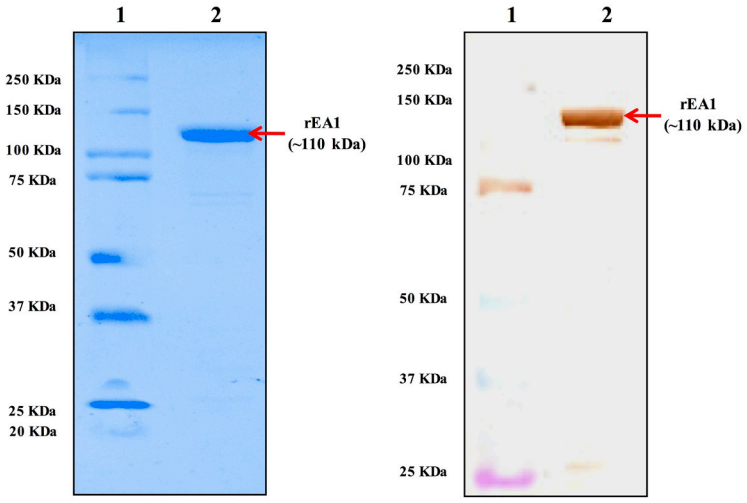

Figure 4. Characterisation of purified rEA1. (a) SDS PAGE profile. Lane 1: Protein markers; lane 2: purified protein. (b) Western blot profile. Lane 1: Protein markers; lane 2: purified protein.

3.7 Development and Evaluation of Antigen Capture ELISA

We have developed antigen capture ELISA for detection of *B. anthracis* spores using polyclonal antibody of rabbit and mouse as capture and revealing antibody, respectively. For this purpose, rEA1 concentration from 200 ng to 0.39 ng/well were tested (Fig. 5). As low as 3.9 ng/ml of rEA could be detected by ELISA. For detection of *B. anthracis* spores, the different number of spores/ ml were tested for the presence of EA1. The ELISA could detect 10^3 spores/ml and so on (Fig. 6(a)). Further, the specificity of ELISA was confirmed using spores of other *Bacillus* species (Fig. 6(b)). No cross reactivity was found with spores of other *Bacillus* species in the developed ELISA.

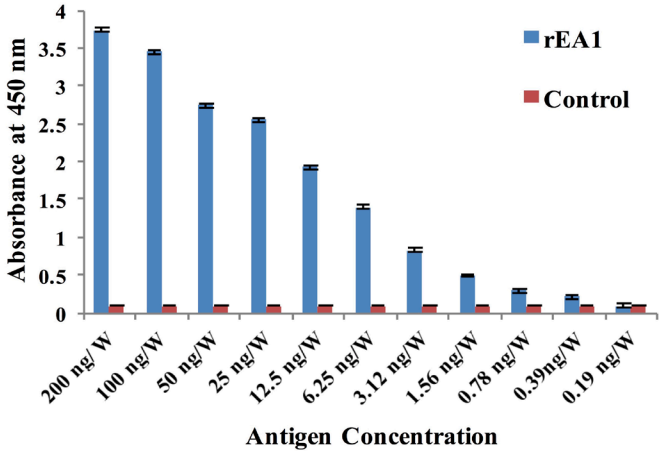

Figure 5. Detection sensitivity of antigen capture ELISA as determined with varying concentration of rEA .

4. DISCUSSION

B. anthracis has long been considered a potential biological warfare agent due to the formation of highly resistant spores. *B. anthracis* spores, either from soil or contaminated animal parts, are the infectious agent of anthrax. One of the major objectives of the present work was to scale up the production of rEA1 protein of *B. anthracis* for development of a sensitive and specific immunological assay for detection of *B. anthracis* spores. Hence, in this study we have done cloning and expression of rEA1 of *B. anthracis*, its scale up, purification and characterisation for development of a detection assay.

The rEA1 was expressed in *E. coli*. About 70 % of the total rEA1 was found in the soluble form. Various culture conditions including inducer concentration, duration and temperature of induction were optimised for enhanced expression of EA1. Varying concentration of IPTG did not affect protein expression at large extent. Induction at 16 °C for 16 h resulted in an increased protein expression as compared to that of 37 °C and 25 °C. These optimised conditions resulted in overall higher level of protein production.

It is well established that the culture media influence the recombinant protein expression levels[21,22]. Among the various growth media studied, TB medium was found to be better as it yielded more biomass and protein expression in

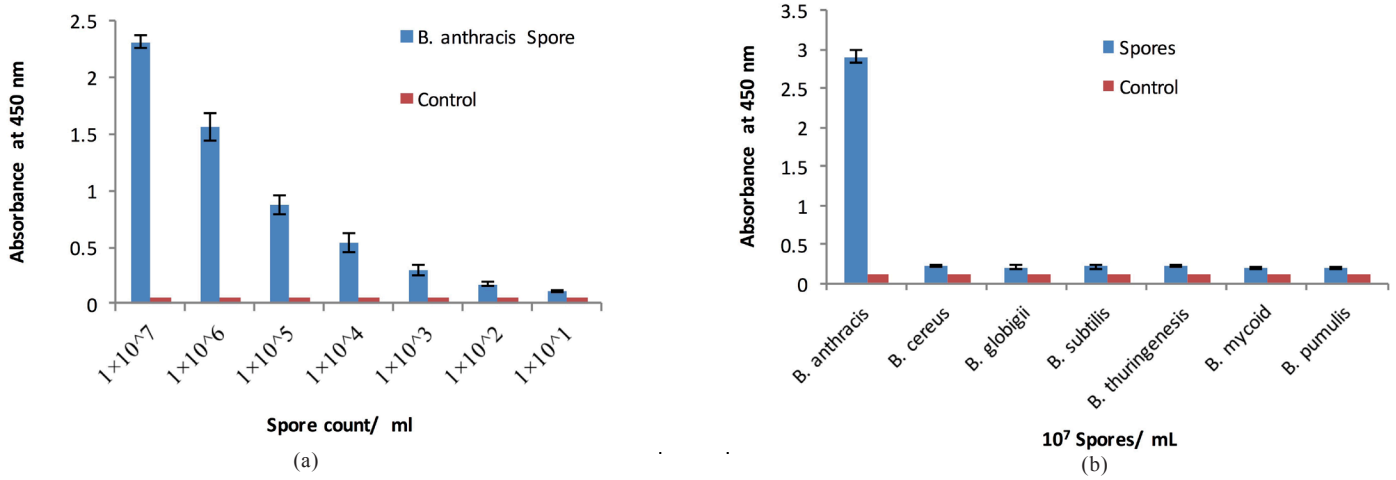

Figure 6. Sensitivity and specificity of sandwich ELISA. (a) Detection sensitivity of *B. anthracis* Sterne spores. (b) Cross reactivity of anti-EA1 polyclonal antibodies with different *Bacillus* species including *B. cereus*, *B. subtilis*, *B. thuringiensis*, *B. globigii*; *B. pumilus, B. mycoides and B. anthracis* Sterne.

comparison to other growth media. Thus, TB medium was used for further scale up process. The higher biomass and protein expression in TB growth medium may be due to presence of glycerol as carbon source and yeast extract as nitrogen source along with salt components.

In this study, scale up of rEA1 was also carried out using bioreactor by batch cultivation. Bulk production of recombinant proteins having the potential of use in detection and diagnostic systems is desirable. Batch fermentation process has been successfully used for bulk production of various proteins. In this report, batch fermentation was done using TB growth medium under optimised conditions. The batch fermentation process resulted in 10.5 g/l of biomass which is greater than biomass resulted at shake flask culture using the LB (2.5 g/l) and TB medium (7.6 g/l).

A single step affinity chromatography purification process was used for rEA1. The affinity chromatography purified protein was further diafiltered. The SDS-PAGE ascertained the molecular weight of purified EA1 as ~110 kDa (Fig. 4(a)). The purified protein was analysed by Western blot using anti-histidine antibodies (Fig. 4(b)) and the results suggested that this protein could be used for specific detection of *B. anthracis*. The protein yield after batch fermentation process using TB medium was also 4-times greater (42.64 mg/l) than shake flask culture using LB medium (10.08 mg/l)

Previous reports have also established that anti-EA1 antibody can be used for detection of *B. anthracis* spores[10,11]. Anti-EA1 monoclonal antibodies have been used even for detection of both, the spores as well as vegetative cells of *B. anthracis* in an earlier study [10]. In this study, rabbit anti-EA polyclonal antibodies were successfully used to detect *B. anthracis* spores (Fig. 4). The ELISA could detect 10^3 spores/ml whereas spores of all the other *Bacillus* species including *B. cereus*, *B. subtilis*, *B. thuringiensis*, *B. globigii, B. pumilus* and *B. mycoides* were found negative for rEA1. Though it is well established that EA1 is not the component of *B. anthracis* spores, yet it is found on the surface of spores in abundance as contaminant during the production of spores[9]. EA1 is not released from the spore surface even after extensive washings during and after spore preparation[10]. Thus, recombinant purified EA1 can be used as a potential biomarker for detection of *B. anthracis* spores.

Compliance with Ethical Standards

All the animal experiments were performed according to the Institutional Animal Ethics Committee (IAEC) vide registration number 37/1999/CPCSEA. The study was also approved by Institutional Biosafety Committee of Defence Research and Development Establishment, DRDO, Ministry of Defence, Government of India wide protocol no: IBSC/12/BT/AKG/22.

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